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13. ABSTRACT (Maximum 200 words)

The purpose of the proposed research is to provide a structural basis for understanding the mechanism of transcription, its regulation, and altered regulation as occurs in tumor cells. The goal of the proposed research is to determine the X-ray structure of RNA Polymerase II in the midst of transcribing RNA from a DNA template.

During the first year of funding, template DNA was shortened to a minimal size without affecting the stability of the paused complex or its efficiency of formation. It was determined that previous platelike crystals are sensitive to physical manipulation, soaking in cryoprotectant or soaking with heavy metal clusters. Crystallization trials of polymerase resulted in new non-platelike crystal forms allowing for the collection of a preliminary data set to 7.5 Angstroms. This is the best elongation complex data set, since previous platelike crystals had mosaic spreads over 1 degree. Recently new crystallization conditions allowed the growing of crystals in cryoprotectant and the generation of yet new crystal forms. Growing in cryoprotectant is a major accomplishment considering the sensitivity of the crystals to manipulations.

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March 5, 1999

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Structural Determination of a Transcribing RNA Polymerase II complex.

Introduction

The goal of the proposed research is to determine the X-ray structures of RNA Polymerase II elongation complexes at atomic resolution and with regulatory proteins. The purpose of the proposed research is to provide a structural basis for understanding the mechanism of transcription, regulation of the process, and altered regulation as occurs in tumor cells. The problem is challenging, since the Polymerase alone comprises 15 polypeptides with a total mass of nearly 600,000 Dalton, and addition of the auxiliary factors adds a further level of complexity.

The transcription mechanism of which RNA Polymerase II is the key player, appears to be universal: the mechanistic details of initiation, RNA chain elongation, and termination, insofar as they have been revealed, are fundamentally the same in bacteria, yeast and higher eukaryotic cells. 6 of the 7 human RNAPII subunits tested were found able to replace their yeast counterparts in vivo (1). Therefore, studies of yeast RNAPII may be expected to reveal general principles of eukaryotic transcription and its regulation. The yeast enzyme is especially suited for 3-D structural analysis because a large amount of pure material can readily be obtained from yeast cell culture. No other cell culture system is able to fulfill such a requirement. Yeast RNA Polymerase lacking subunits 4 and 7 (D4/7) were originally used for 2-dimentional crystallography since they were more homogenous than the wild type enzyme (2,3).

Despite the many subunits, eukaryotic RNA Polymerase II enzymes are alone incapable of recognizing a promoter and initiating transcription. A number of accessory factors, referred to as general transcription or general initiation factors, are required (for reviews see ref. 4, 5, 6). Regulation of Polymerase transcription has recently been attributed to a large multi-subunit complex which was termed mediator (7). Mediator complex co-purifies with RNA-Polymerase II in a complex known as holoenzyme. Although initially found in yeast, the human counterpart with subunit homology has been shown to exist and is also involved in regulation of transcription (8).

In breast cancer research there are two major routes of study. The first is to develop methods to directly intervene and eradicate tumor cells. These can be by invasive or non- invasive means. A second route of study is in advancing our knowledge of the disorder itself. The current research is of this nature. Cancer cells are different than normal cells in that they have altered regulation. The key point of regulation on the cellular level is that of transcription. Indeed mutations in tumor suppressor genes such as P^{53} and inherited mutations in the breast and ovarian cancer susceptibility gene, BRCA1, are directly associated with breast cancer.

The path by which mutations are capable of altering cellular traits is by affecting regulation of specific genes either at initiation or elongation of RNA Polymerase II. During elongation, RNA Polymerase II pauses on its transcript. Proteins such as TFIIS, can regulate the amount of the read through. Indeed, an additional elongation factor, Elongin (SIII), has been shown to be a target of the VHL tumor suppressor protein and able to directly regulate its function (9). Mutations in the VHL gene predispose individuals to a variety of tumors (10). This is a clear case of point mutations in a gene, directly affecting the regulatory mechanism

In the case of breast cancer there has been growing evidence that altered regulation occurs on the level of RNA Polymerase II transcription initiation as well as elongation. Recently, P^{53} has been shown to regulate CAK kinase activity. CAK kinase is a component of the basal transcription factor TFIIH found to be necessary for CTD phosphorylation of RNA Polymerase II in order to allow elongation of the RNA transcript (11). Another example is that of BRCA1 which was found to be a component of the RNA Polymerase II holoenzyme complex (12). It was further shown to, activate transcription when linked with a DNA-binding domain (13).

The most efficient means of generating RNA Polymerase II elongation complexes is with the aid of tailed oligonucleotide templates. Initiation on a single strand protruding from the 3'-end of duplex DNA does not require accessory factors and allows for highly efficient generation of functional elongation complexes (9). Such a "tailed" template may be viewed as half of an unwound "bubble", which occurs at the active site of RNA Polymerase molecules during transcription. Consistent with this idea, transcription starts within the single stranded region, about three bases from the junction with duplex DNA, in both tailed templates and in the unwound bubble of an elongation complex (10). At least two possible paused complexes can exist. The first is halted due to the lack of a single nucleotide such as UTP (11). The second is arrested even in the presence of all nucleotides, due to the DNA structure arising from its primary sequence (12). Although structural determination of the ternary complex in a functional state until date has not been shown, use of tailed templates has allowed the applicant to develop a system with appropriate templates for the generation, purification and crystallization of this complex (13).

Elongation complexes were "halted" on tailed templates by transcription in the absence of UTP, so that the Polymerase halted when the first T residue in the template was reached. The halted complexes generated on the tailed templates are advantageous for crystallization because of their uniformity in content of DNA and RNA sequences (14).

Before acquiring this fellowship, platelike crystals of the delta 4/7 enzyme were successfully grown and diffracted (14). Diffraction data taken on station F-1 at CHESS were complete to 6.4 Å and extended in some directions to 3.5 Å. Few crystals survived soaking and freezing, however, and the mosaic spread of the diffraction was high (greater than 1°).

Body

1. Specific aims as they appear in the original proposal

1. X-ray structure determination of RNA Polymerase II at 6 Å resolution. Heavy atom clusters are being employed for phase determination at this resolution, and results from electron microscope crystallography of unstained specimens in progress may provide additional phase constraints. A 6 Å structure should prove useful for locating the positions of bound DNA and RNA and for locating positions of discrete heavy atoms in the subsequent high resolution phasing process.

2. X-ray structure determination of ternary complex at 3.5 Å resolution. Heavy atom reagents that are capable of specifically binding DNA will be used to prepare isomorphous derivative crystals. Phases from the low resolution structure will be used in cross-difference Fourier calculations to identify such heavy atom sites. Additional model-building may be aided by the structural features revealed by electron microscopy crystallography. In addition preliminary studies with Tn clusters seems successful and promising as some reveal peaks on Patterson maps. This data will be employed for initial phasing.

3. Determining the structure of DNA sequences in the ternary complex caused by intrinsic pausing, a point of cellular regulation of elongation complexes. Currently available templates allow for the generation of such complexes. This would allow for analysis and comparison of DNA primary sequence with its structure in the paused complex, providing possible information on elongation control points in innumerable genes based on the comparison of primary DNA sequence data with structural data from intrinsically paused complexes.

4. Co-crystallization of ternary complex with TFIIS, one of the proteins that regulates elongation at pause sites.

2. Key decision to search for new crystal forms.

Previously, co-workers in the Kornberg laboratory in Stanford University have been crystallized and diffracted crystals of RNA Polymerase II (D4/7) and collected data sets to 3.5 Å. Similar crystallization conditions applied to elongation complexes with DNA and RNA allowed for poorly diffracting crystals, with a diffraction limit of 6.0 Å and a high mosaic spread. Furthermore when soaked with heavy metals and/or cryoprotectant they cracked. A key decision to search for new crystal forms was then implemented. Various oligonucleotide templates were studied in an attempt to improve the quality of the crystals but only plate crystals were observed. In last years report wild type RNA Polymerase II (with subunits 4 and 7) was crystallized as an elongation complex. Improved conditions were found by growing crystals in cryoprotectant (PEG 400). Although consistent diffraction was achieved and a data set was taken, the resolution limit was only 7.5 Å. These crystal growth conditions were applied this year to the D4/7 Polymerase. Since they are grown in cryoprotectant, it would withhold the need to soak plate crystals and might have allowed for better

diffraction. To our dismay, PEG 400 conditions resulted in needles, and did not allow for the growth of D4/7 crystals.

As a result other conditions were screened over a period of 5 months. In most cases either needle-like crystals were formed, or plate crystals with the same inherent weaknesses as the original crystals. Another approach to finding better crystal forms involves adding components that may alter the protein- protein interactions. These approaches were taken in paragraphs 2 and 3 below. To understand the effects of adding additional components to Polymerase, a set of oligonucleotide templates was developed and employed for biochemical analysis (see below).

Figure 1. Templates used in generating elongation complexes

1Pause

```
          AAGACCAGGCATTTTTTCTTTTTGCGGAAGCTA
C15-      TTCTGGTCCGTAAAAAAGAAAAACGCCTTCGAT
```

3Pause

```
               AACACCAGCGATTTCGGGGAAGAACAAGCAAAC
C12-      GTGTTGTGGTCGCTAAAGCCCCTTCTTGTTCGTTTG
```

4Pause

```
          AAGCCCACCCATTTCGGGGAAGAACAAGCAAAC
C15-      TTCGGGTGGGTAAAGCCCCTTCTTGTTCGTTTG
```

9Pause

```
          AAGACCAGGCATTTTTTCTTGTTGCGGAAGGGG
C12-      TTCTGGTCCGTAAAAAAGAACAACGCCTTC
```

CM

```
          AAGACCAGGCATTTTTTCTTGTTGCGGAAGGGG
C12-      TTCTGGTCCGTAAAAAAGAACAACGCCTTC
            *     *               *  *  *  *
```

TM2

```
          AAGACCAGGCATTTTTTCTTGTTGCGGAAGGGG
CCCCCCCCCCCCTTCTGGTCCGTAAAAAAGAACAACGCCTTC
*******
```

Figure 1. Tailed template DNA's for formation of paused RNA Polymerase II elongation complexes. Stars represent nucleotides containing bromide. The 9Pause template sequence is currently employed in generation of elongation crystals.

3. Use of RNA Polymerase II - TFIIS co-crystals to improve diffraction

In objective number 4 of this proposal we suggested that we would attempt to co-crystallize RNA Polymerase II with the elongation factor TFIIS. This factor is biochemically well characterized. Itself, it lacks any enzymatic activity but it can cause Polymerase to cut the RNA chain. This is especially important since during transcription nucleotide sequences which cause RNA Polymerase to arrest are encountered and transcription is paused. TFIIS allows for the resumption of transcription and is therefore a key regulatory protein.

Another technically important aspect of using it for co-crystals was in the possibility of improving crystal quality and improving of diffraction quality and resolution. In elongation complexes of wild type and D4/7, anisotropic diffraction was observed with poorest diffraction in the c direction. The idea was then to add an RNA Polymerase II binding polypeptide that could increase/alter contacts in this direction. Initially RNA Polymerase II D4/7 -TFIIS co-crystals were grown and resulted in plate crystals. These were taken to SSRL at beamline 9-I and to our surprise diffracted very well. Some difficulties arose in that the MAR scanner was not functioning properly and the crystal slipped out of the beam towards the end. The best crystal was saved for the next time that synchrotron radiation is made available.

Figure 2. Crystallographic data for yeast RNA Polymerase II (pol II) and its complexes

Molecule	Unit Cell	Space Group	X-ray Source	Resolution Limit (Å)	Rsymm	<I/ sigma >	Complete-ness
pol II	a = 131.3 b = 223.7 c = 368.9	I222	SSRL BL7-1	3.0	9.9 % for 50.0-4.0 Å	30.0	75% for 50-4.0 Å
	a = 211.4 b = 222.3 c = 321.6	I222 or I212121	SSRL BL7-1	5.0	7.5% for 50.0-5.0 Å		98% for 50.0-5.0 Å
Transcription elongation complex	a = 174.9 b = 222.2 c = 196.5 b = 103.3°	C2	CHESS F-1	6.0	6.1% for 70-6.4 Å	6.2	71% for 70-6.4
Wild-type pol II	a = 218.9 b = 390.5 c = 279.8	C222	SSRL BL 1-5	6.0	6.3% for 50-6.0 Å	4.7	95% to 6.0 Å
*Wild-type elongation complex	a = 219.8 b = 389.1 c = 272.0	C222	SSRL BL 1-5	6.0	7.6% for 23.7-7.5 Å	7.2	74% to 7.5 Å
*TFIIS- pol II	a = 170.9 b = 218.9 c = 193.9 b = 99.9°	C2	SSRL BL 9-1	3.34 (Edge of plate, perhaps better)	7.6% for 16.0-3.6 Å	7.3	60% to 3.6 Å

* Performed under the current study.

Despite these drawbacks 60 percent of the data set was taken and diffraction reached the edge of the plate at 3.34 Å. These crystals are very similar to the elongation complex crystals. Firstly they are morphologically plate crystals and have similar though not identical cell units (see table 2 above). The improved diffraction caused by the presence of TFIIS opens the door to high-resolution diffraction. We are now going to generate elongation-TFIIS crystals. The improved crystal form may allow for elongation complexes to yield high-resolution data and in addition, will represent the first structure of a regulated elongation complex. Since the wild type enzyme is also anisotropic and also diffracts poorly, TFIIS will be co-crystallized with this enzyme as well as the with wild type elongation form. As such, the possibility of fulfilling a high-resolution structure in the last year of this application has become a reality.

Figure 3.

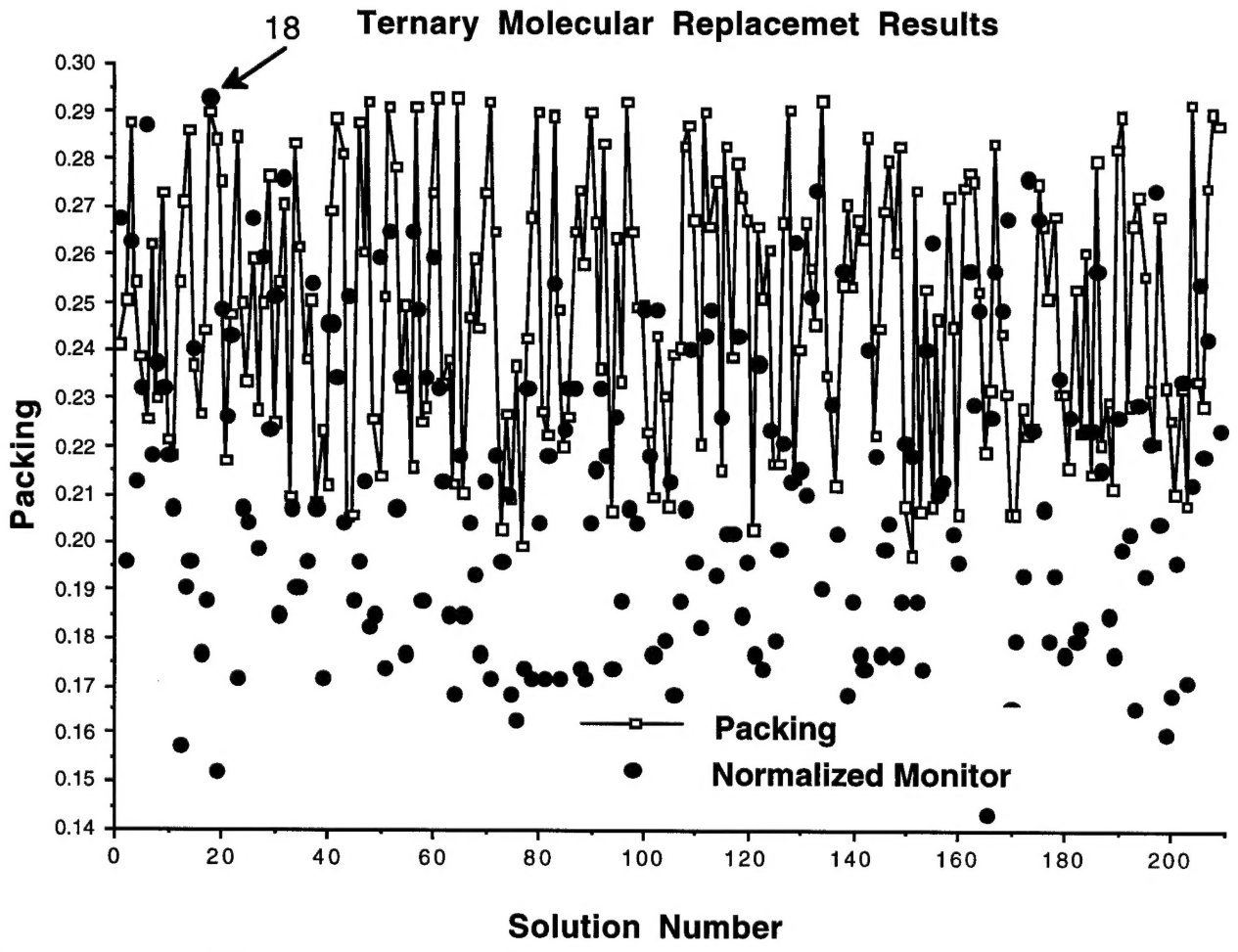

Figure 3 Ternary molecular replacement results were from CNS version 4.0 and employed the structure of RNA polymerase II from the previous 3D reconstructed data. The packing value represents volume in the unit cell, which in this case is optimal at around 29%. Monitor values represent correlation values of Patterson maps employed. These values were scaled to the packing values for the purpose of this presentation. Only solution 18 has both a high packing and monitor value.

4. Molecular replacement of previous low-resolution data to solve the structure of elongation complexes.

When soaking ternary plate crystals in cryoprotectant or with heavy metals they tend to crack. The strategy was then to collect native data sets from these crystals and apply molecular replacement with data achieved from Polymerase alone not in elongation complex. Recently, a Co-worker arrived at a good set of phases for Polymerase to 5 Å. For the first time we are able to perform molecular replacement of elongation complex data using the known polymerase structure. This was the primary goal of this effort. To find the orientation and location of the asymmetric unit in the elongation complex we have begun two X-PLOR (CNS) rotation analysis jobs using a PDB formatted model of the D4/7 enzyme generated from the program BONES. In addition molecular replacement will be performed with the original densities using the AMORE programs.

To verify results, molecular replacement has been completed with the electron microscope 3-D structure. As can be seen in figure 2, there was only one solution that had a high monitor score (correlation score) and high packing score. This solution can not only be used to verify the results of X-PLOR and AMORE, but can also be used to fit the known Polymerase structure into and generate a new set of phases. The molecular replacement should yield results in the near future, at about the time of submission of this report.

4. Use of heavy metal nucleotides in generating crystals for nucleic acid localization

It is unclear if the initial elongation structure at low resolution (~6A), will allow for the visualization of RNA on the surface of the enzyme. To overcome this difficulty, elongation complexes with Mercury-CTP instead of CTP were generated with template 9Pause. This would allow for localization of the RNA chain. The resultant RNA chain would have two adjacent C residues labeled with mercury, followed by one more, four bases away. This will also allow for the determination of the direction of RNA synthesis even if the RNA is not represented by clear density in the low-resolution map. Producing elongation complex with mercury CTP requires DTT since reducing agents that do not react with mercury such as TCEP do not allow for enzymatic activity (figure 4). This is because mercury in the nucleotide can react with the protein if not coupled to a reducing agent. Employing thiourea as reducing agent instead of DTT or TCEP also allowed for generation with 9Pause of paused complexes (see figure 5).

Figure 4. Mercury-CTP incorporation into elongation complexes.

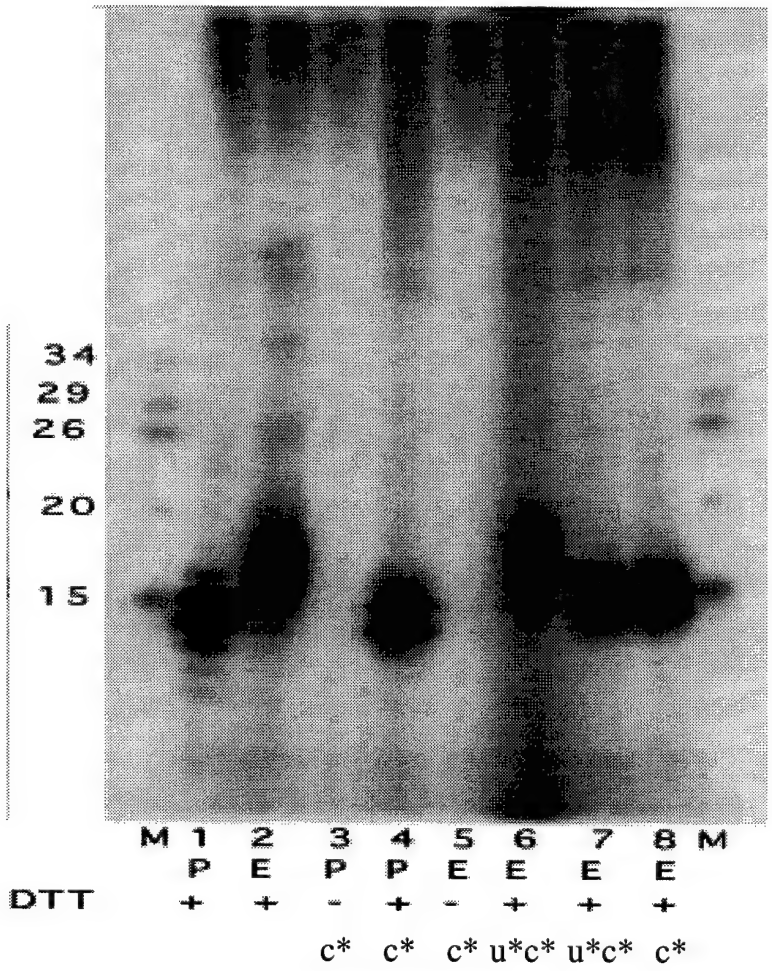

Figure 4. Template 1Pause was employed for all samples. RNA was generated and electrophoresis accomplished as previously described (?). P32 labeled GTP was incorporated into the RNA. P – paused by withholding UTP. E – Elongated in the presence of all four RNA nucleotide substrates. M – is a mixture of oligodeoxynucleotides with sizes of 15, 20, 26, 29 and 34 bases. In samples where DTT was not added, TCEP, which does not react with mercury was used. Samples 3-8 contained mercury-CTP at 60 uM final concentration in the reaction mix and samples 7 and 8 (labeled u*) contained additionally 60 uM and 25 uM mercury- UTP respectively.

Employing template 1Pause in the presence of reducing agent allowed for the efficient incorporation of the mercury-CTP. The efficiency of incorporation though depends on the specific sequence of the template and/or of the RNA generated such that template 3Pause and 4Pause could only inefficiently generate elongation complexes when mercury- CTP was employed (lanes 6-13 figure 5). Templates 1Pause and 9Pause (see template list) give good yields with mercury- CTP and therefore the shorter template 9Pause will continue to be used the template for elongation complexes. Elongation crystals were then generated having mercury in the RNA. Initially, crystals appeared to be identical to the old plate form, growing in 4-7 days and having a typical hexagonal plate structure. These crystals were once again plagued by

their poor quality. Several crystals however were observed to grow in 12-20 days. These were also plate crystals but when soaked with cryoprotectant and heavy metal (Tn clusters) did not crack. 8 of such crystals are now at hand for the next synchrotron run. The best of these crystals will be used for collection of a MAD data set, since they are labeled with both Tn and Mercury. This data will also corroborate results from the molecular replacement and yield valuable information on the location of the RNA chain.

Figure 5. Mercury-nucleotide incorporation and template specificity in generating elongation complexes.

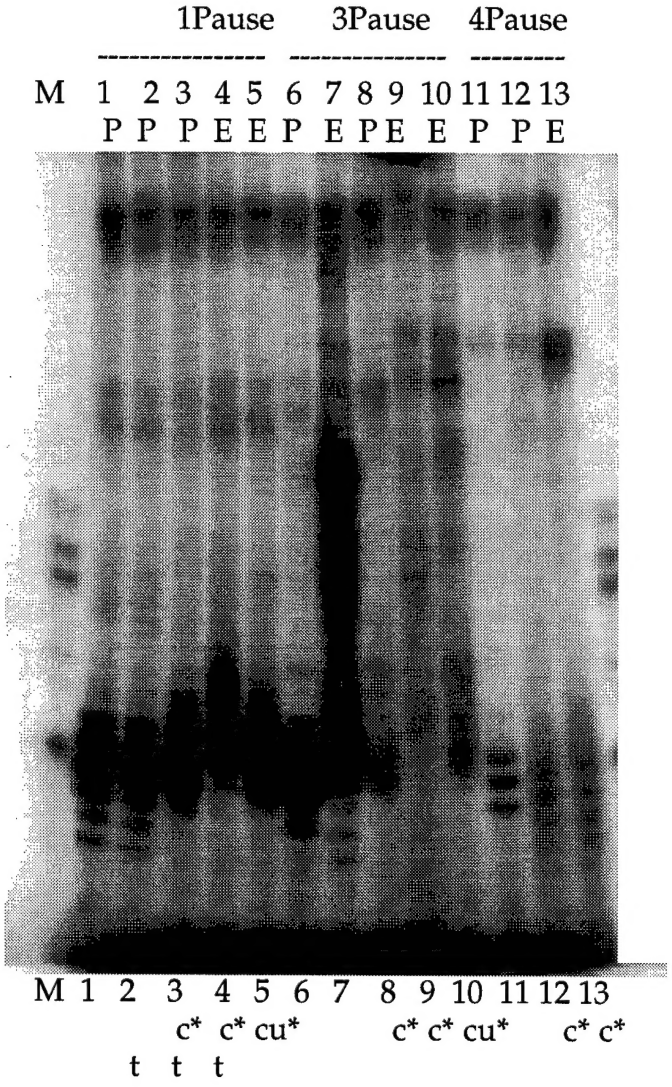

Figure 5. Templates 1,3 and 4Pause were employed as indicated. RNA was generated and electrophoresis accomplished as previously described (14). P32 labeled GTP was incorporated into the RNA as in figure 4. P – paused by withholding UTP. E – Elongated in the presence of all four RNA nucleotide substrates. M – is a mixture of oligodeoxynucleotides with sizes of 15, 20, 26, 29 and 34 bases. DTT was employed in all samples excluding those labeled with t. Those employed thiourea as reducing agent. Samples contained mercury-CTP (c*) at 600 uM final concentration in the reaction mix and samples 7 and 8 (u*) contained in addition 60 uM mercury-UTP.

Interestingly, although mercury-CTP was efficiently incorporated into template 1Pause, mercury -UTP was not well tolerated and allowed for very poor elongation or no elongation at all (Lanes 7 and 8 figure 4, lanes 4 and 5 figure 5). Template 4Pause did not even tolerate mercury CTP.

In addition to developing a tool for localizing the RNA at low resolution, an initial attempt at labeling the DNA was made. Two oligonucleotides were synthesized with bromine in cytosine bases. Indeed not only are these functional as templates but they can be used in conjunction with mercury labeled RNA (figure 6). The only drawback is in the reduced yields of elongation complexes. Initial crystal trials resulted in microcrystals for a bromide template with bromide located in the single stranded tail (template TM2), and no crystals when bromide was incorporated into the body of the template (template CM), even though both serve as functional templates.

Figure 6. Mercury-nucleotide incorporation and template specificity in generating elongation complexes.

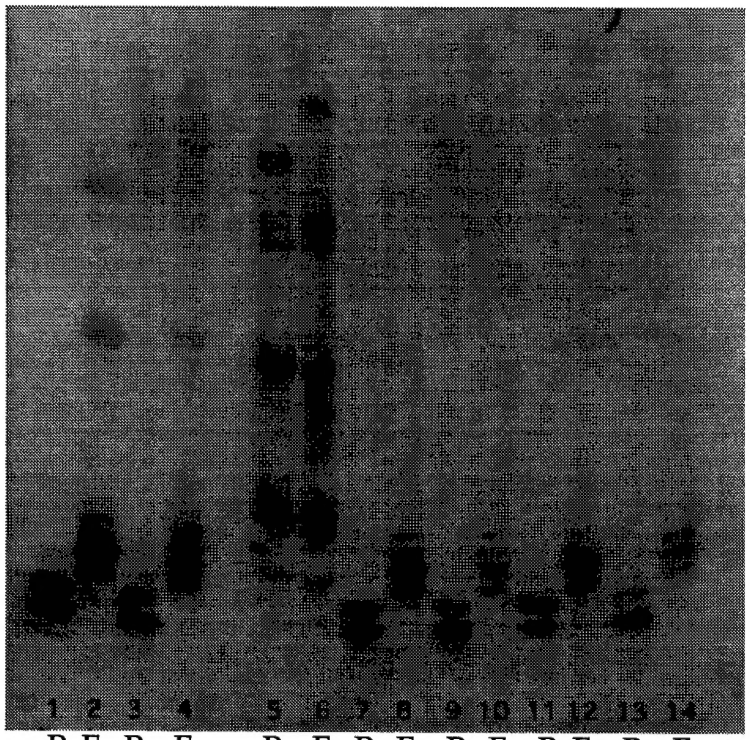

Figure 6. Symbols are as in figures 4 and 5. Template Jfu has an 18 base single stranded region and an 18 base pair double stranded region. Such short templates are inefficient at synthesizing RNA chains. Note that all other templates functioned well though with diminished yields of elongation complex.

Conclusions

Objectives of this proposal originally included employing platelike crystals of delta 4/7 enzyme and heavy metal clusters for phasing for low resolution structure determination (~6A). In the second and third years the goals were to achieve high resolution data of the elongation complex and co-crystallize Polymerase with TFIIS.

The current stage is indeed a crucial one. Firstly, we are now in the midst of molecular replacement to achieve the elongation complex structure to low resolution. In addition crystals of elongation complex with mercury labeled RNA were generated and may allow the direct localization of the RNA before a high resolution structure is generated. Indeed, it was found that template 9Pause can be used to generate elongation complexes that contain mercury in the RNA strand or Bromide in the DNA. Not all templates are suitable for this purpose. Secondly, TFIIS- Polymerase co-crystals were generated that give high resolution diffraction. This is another powerful discovery not only because it fulfills the desires of the original proposal of generating Polymerase-TFIIS co-crystals, but also because it has a good chance of allowing us to achieve similar elongation co-crystals. Indeed we are now generating Polymerase (wt and D4/7) elongation- TFIIS co-crystals. Considering the difficulties involved in this project, we feel that success is finally at hand and that our last year of support will by far be the most productive and rewarding.

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